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(54) **CUTINASE VARIANTS AND
POLYNUCLEOTIDES ENCODING SAME**

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See application file for complete search history.

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(57) **ABSTRACT**

The present invention relates to cutinase variants. The present invention also relates to polynucleotides encoding the variants; nucleic acid constructs, vectors, and host cells comprising the polynucleotides; and methods of using the variants.

15 Claims, No Drawings

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CUTINASE VARIANTS AND POLYNUCLEOTIDES ENCODING SAME

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a 35 U.S.C. 371 national application of PCT/EP2013/059040 filed Apr. 30, 2013, which claims priority or the benefit under 35 U.S.C. 119 of Indian application no. 1895/CHE/2012 filed May 14, 2012 and U.S. provisional application No. 61/667,676 filed Jul. 3, 2012, the contents of which are fully incorporated herein by reference.

REFERENCE TO A SEQUENCE LISTING

This application contains a Sequence Listing in computer readable form, which is incorporated herein by reference.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to cutinase variants, polynucleotides encoding the variants, methods of producing the variants, and methods of using the variants.

2. Description of the Related Art

Cutinases are lipolytic enzymes capable of hydrolyzing the substrate cutin. Cutinases are known from various fungi (P. E. Kolattukudy in "Lipases", Ed. B. Borgstrom and H. L. Brockman, Elsevier 1984, 471-504) such as cutinases from *Fusarium solani pisi* (S. Longhi et al., Journal of Molecular Biology, 268 (4), 779-799 (1997)) and *Humicola insolens* (U.S. Pat. No. 5,827,719). Variants of the cutinase from *Humicola insolens* are known from e.g. WO00/34450.

Cutinases may be used in the polymerization of acrylates. Acrylates are industrially important compounds that have a wide range of commercial applications. The α -double bond present in these molecules can be used for polymerization to yield final products such as paints, plastics and adhesives. Traditionally, esters of acrylic acid and methacrylic acid are made chemically at elevated temperature, with polymerization inhibitors and with sulphuric acid as catalyst. Enzymatic transacylation of acrylates is a "green" option to traditional chemistry and could provide sustainable routes to acrylic esters starting from renewable sources such as glucose and lactate.

The present invention provides variants of a parent cutinase with improved properties compared to its parent, in particular variants having increased transacylation activity.

SUMMARY OF THE INVENTION

The present invention relates to an isolated variant of a parent cutinase comprising a substitution at one or more positions corresponding to amino acid residue 31, 36, 45, 52, 70, 120, 143, 167, and 174 of the mature polypeptide of SEQ ID NO: 2 selected from the group consisting of: P31A,R,N,D,C,Q,E,G,H,I,L,K,M,F,S,T,W,Y,V; I36A,R,N,D,C,Q,E,G,H,L,K,M,F,P,S,T,W,Y,V; G45A,R,N,D,C,Q,E,H,I,L,K,M,F,P,S,T,W,Y,V; N52E; F70A,R,N,D,C,Q,E,G,H,I,L,K,M,P,S,T,W,Y,V; G120S; G143A,R,N,D,C,Q,E,H,I,L,K,M,F,P,S,T,W,Y,V; L167A; and L174N,Q, and which variant has cutinase activity.

The present invention also relates to isolated polynucleotides encoding the variants; nucleic acid constructs, vectors, and host cells comprising the polynucleotides; and methods of producing the variants.

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The present invention also relates to methods modifying a surface, converting cutins in plant biomass to chemical feedstocks, and recycling a polyester.

Definitions Cutinase: The term "Cutinase" means a lipolytic enzyme with cutinase activity (EC3.1.1.74) that catalyzes the reaction: $\text{Cutin} + \text{H}_2\text{O} \rightleftharpoons \text{Cutin monomers}$. Lipolytic enzymes capable of hydrolyzing the substrate cutin are classified as EC3.1.1.74 according to Enzyme Nomenclature available at <http://www.chem.qmw.ac.uk/iubmb/enzyme>. For purposes of the present invention, cutinase activity is determined according to the procedure described in the Examples. In one aspect, the variants of the present invention have at least 20%, e.g., at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 100% of the cutinase activity of the mature polypeptide of SEQ ID NO: 2. Allelic variant: The term "allelic variant" means any of two or more alternative forms of a gene occupying the same chromosomal locus. Allelic variation arises naturally through mutation, and may result in polymorphism within populations. Gene mutations can be silent (no change in the encoded polypeptide) or may encode polypeptides having altered amino acid sequences. An allelic variant of a polypeptide is a polypeptide encoded by an allelic variant of a gene.

cDNA: The term "cDNA" means a DNA molecule that can be prepared by reverse transcription from a mature, spliced, mRNA molecule obtained from a eukaryotic or prokaryotic cell. cDNA lacks intron sequences that may be present in the corresponding genomic DNA. The initial, primary RNA transcript is a precursor to mRNA that is processed through a series of steps, including splicing, before appearing as mature spliced mRNA.

Coding sequence: The term "coding sequence" means a polynucleotide, which directly specifies the amino acid sequence of a variant. The boundaries of the coding sequence are generally determined by an open reading frame, which begins with a start codon such as ATG, GTG or TTG and ends with a stop codon such as TAA, TAG, or TGA. The coding sequence may be a genomic DNA, cDNA, synthetic DNA, or a combination thereof.

Control sequences: The term "control sequences" means nucleic acid sequences necessary for expression of a polynucleotide encoding a variant of the present invention. Each control sequence may be native (i.e., from the same gene) or foreign (i.e., from a different gene) to the polynucleotide encoding the variant or native or foreign to each other. Such control sequences include, but are not limited to, a leader, polyadenylation sequence, propeptide sequence, promoter, signal peptide sequence, and transcription terminator. At a minimum, the control sequences include a promoter, and transcriptional and translational stop signals. The control sequences may be provided with linkers for the purpose of introducing specific restriction sites facilitating ligation of the control sequences with the coding region of the polynucleotide encoding a variant.

Expression: The term "expression" includes any step involved in the production of a variant including, but not limited to, transcription, post-transcriptional modification, translation, post-translational modification, and secretion.

Expression vector: The term "expression vector" means a linear or circular DNA molecule that comprises a polynucleotide encoding a variant and is operably linked to control sequences that provide for its expression.

Fragment: The term "fragment" means a polypeptide having one or more (e.g., several) amino acids absent from

the amino and/or carboxyl terminus of a mature polypeptide; wherein the fragment has cutinase activity. In one aspect the number of amino acids of the fragment is at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 85%, at least 90%, or at least 95% of the number of amino acids of the mature polypeptide.

Host cell: The term "host cell" means any cell type that is susceptible to transformation, transfection, transduction, or the like with a nucleic acid construct or expression vector comprising a polynucleotide of the present invention. The term "host cell" encompasses any progeny of a parent cell that is not identical to the parent cell due to mutations that occur during replication.

Improved property: The term "improved property" means a characteristic associated with a variant that is improved compared to the parent. Such improved property includes, but is not limited to, catalytic efficiency, catalytic rate, specific activity, substrate binding, substrate cleavage, substrate specificity.

Isolated: The term "isolated" means a substance in a form or environment which does not occur in nature. Non-limiting examples of isolated substances include (1) any non-naturally occurring substance, (2) any substance including, but not limited to, any enzyme, variant, nucleic acid, protein, peptide or cofactor, that is at least partially removed from one or more or all of the naturally occurring constituents with which it is associated in nature; (3) any substance modified by the hand of man relative to that substance found in nature; or (4) any substance modified by increasing the amount of the substance relative to other components with which it is naturally associated (e.g., multiple copies of a gene encoding the substance; use of a stronger promoter than the promoter naturally associated with the gene encoding the substance). An isolated substance may be present in a fermentation broth sample.

Mature polypeptide: The term "mature polypeptide" means a polypeptide in its final form following translation and any post-translational modifications, such as N-terminal processing, C-terminal truncation, glycosylation, phosphorylation, etc. In one aspect, the mature polypeptide is amino acids 1 to 194 of SEQ ID NO: 2. It is known in the art that a host cell may produce a mixture of two or more different mature polypeptides (i.e., with a different C-terminal and/or N-terminal amino acid) expressed by the same polynucleotide.

Mature polypeptide coding sequence: The term "mature polypeptide coding sequence" means a polynucleotide that encodes a mature polypeptide having cutinase activity. In one aspect, the mature polypeptide coding sequence is nucleotides 109 to 690 of SEQ ID NO: 1.

Mutant: The term "mutant" means a polynucleotide encoding a variant.

Nucleic acid construct: The term "nucleic acid construct" means a nucleic acid molecule, either single- or double-stranded, which is isolated from a naturally occurring gene or is modified to contain segments of nucleic acids in a manner that would not otherwise exist in nature or which is synthetic, which comprises one or more control sequences.

Operably linked: The term "operably linked" means a configuration in which a control sequence is placed at an appropriate position relative to the coding sequence of a polynucleotide such that the control sequence directs expression of the coding sequence.

Parent or parent cutinase: The term "parent" or "parent cutinase" means a cutinase to which an alteration is made to produce the enzyme variants of the present invention. The

parent may be a naturally occurring (wild-type) polypeptide or a variant or fragment thereof.

Sequence identity: The relatedness between two amino acid sequences or between two nucleotide sequences is described by the parameter "sequence identity".

For purposes of the present invention, the sequence identity between two amino acid sequences is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, *J. Mol. Biol.* 48: 443-453) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice et al., 2000, *Trends Genet.* 16: 276-277), preferably version 5.0.0 or later. The parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the EBLOSUM62 (EMBOSS version of BLOSUM62) substitution matrix. The output of Needle labeled "longest identity" (obtained using the—nobrief option) is used as the percent identity and is calculated as follows:

$$\frac{(\text{Identical Residues} \times 100) / (\text{Length of Alignment} - \text{Total Number of Gaps in Alignment})}{20}$$

For purposes of the present invention, the sequence identity between two deoxyribonucleotide sequences is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, *supra*) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice et al., 2000, *supra*), preferably version 5.0.0 or later. The parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the EDNAFULL (EMBOSS version of NCBI NUC4.4) substitution matrix. The output of Needle labeled "longest identity" (obtained using the—nobrief option) is used as the percent identity and is calculated as follows:

$$\frac{(\text{Identical Deoxyribonucleotides} \times 100) / (\text{Length of Alignment} - \text{Total Number of Gaps in Alignment})}{35}$$

Subsequence: The term "subsequence" means a polynucleotide having one or more (e.g., several) nucleotides absent from the 5' and/or 3' end of a mature polypeptide coding sequence; wherein the subsequence encodes a fragment having cutinase activity. In one aspect, a subsequence contains at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 85%, at least 90%, or at least 95% of the number of nucleotides encoding the mature polypeptide.

Variant: The term "variant" means a polypeptide having cutinase activity comprising a substitution at one or more (e.g., several) positions. A substitution means replacement of the amino acid occupying a position with a different amino acid. The variants of the present invention have at least 20%, e.g., at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75% at least 80%, at least 85%, at least 90%, at least 95%, or at least 100% of the cutinase activity of the mature polypeptide of SEQ ID NO: 2.

Wild-type cutinase: The term "wild-type" cutinase means a cutinase expressed by a naturally occurring microorganism, such as a bacterium, yeast, or filamentous fungus found in nature.

Conventions for Designation of Variants

For purposes of the present invention, the mature polypeptide disclosed in SEQ ID NO: 2 is used to determine the corresponding amino acid residue in another cutinase. The amino acid sequence of another cutinase is aligned with the mature polypeptide disclosed in SEQ ID NO: 2, and based on the alignment, the amino acid position number corresponding to any amino acid residue in the mature polypep-

tion disclosed in SEQ ID NO: 2 is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, *J. Mol. Biol.* 48: 443-453) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice et al., 2000, *Trends Genet.* 16: 276-277), preferably version 5.0.0 or later. The parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the EBLOSUM62 (EMBOSS version of BLOSUM62) substitution matrix.

Identification of the corresponding amino acid residue in another cutinase can be determined by an alignment of multiple polypeptide sequences using several computer programs including, but not limited to, MUSCLE (multiple sequence comparison by log-expectation; version 3.5 or later; Edgar, 2004, *Nucleic Acids Research* 32: 1792-1797), MAFFT (version 6.857 or later; Katoh and Kuma, 2002, *Nucleic Acids Research* 30: 3059-3066; Katoh et al., 2005, *Nucleic Acids Research* 33: 511-518; Katoh and Toh, 2007, *Bioinformatics* 23: 372-374; Katoh et al., 2009, *Methods in Molecular Biology* 537: 39-64; Katoh and Toh, 2010, *Bioinformatics* 26: 1899-1900), and EMBOSS EMMA employing ClustalW (1.83 or later; Thompson et al., 1994, *Nucleic Acids Research* 22: 4673-4680), using their respective default parameters.

When the other enzyme has diverged from the mature polypeptide of SEQ ID NO: 2 such that traditional sequence-based comparison fails to detect their relationship (Lindahl and Elofsson, 2000, *J. Mol. Biol.* 295: 613-615), other pairwise sequence comparison algorithms can be used. Greater sensitivity in sequence-based searching can be attained using search programs that utilize probabilistic representations of polypeptide families (profiles) to search databases. For example, the PSI-BLAST program generates profiles through an iterative database search process and is capable of detecting remote homologs (Atschul et al., 1997, *Nucleic Acids Res.* 25: 3389-3402). Even greater sensitivity can be achieved if the family or superfamily for the polypeptide has one or more representatives in the protein structure databases. Programs such as GenTHREADER (Jones, 1999, *J. Mol. Biol.* 287: 797-815; McGuffin and Jones, 2003, *Bioinformatics* 19: 874-881) utilize information from a variety of sources (PSI-BLAST, secondary structure prediction, structural alignment profiles, and solvation potentials) as input to a neural network that predicts the structural fold for a query sequence. Similarly, the method of Gough et al., 2000, *J. Mol. Biol.* 313: 903-919, can be used to align a sequence of unknown structure with the superfamily models present in the SCOP database. These alignments can in turn be used to generate homology models for the polypeptide, and such models can be assessed for accuracy using a variety of tools developed for that purpose.

For proteins of known structure, several tools and resources are available for retrieving and generating structural alignments. For example the SCOP superfamilies of proteins have been structurally aligned, and those alignments are accessible and downloadable. Two or more protein structures can be aligned using a variety of algorithms such as the distance alignment matrix (Holm and Sander, 1998, *Proteins* 33: 88-96) or combinatorial extension (Shindyalov and Bourne, 1998, *Protein Engineering* 11: 739-747), and implementation of these algorithms can additionally be utilized to query structure databases with a structure of interest in order to discover possible structural homologs (e.g., Holm and Park, 2000, *Bioinformatics* 16: 566-567).

In describing the variants of the present invention, the nomenclature described below is adapted for ease of refer-

ence. The accepted IUPAC single letter or three letter amino acid abbreviation is employed.

Substitutions. For an amino acid substitution, the following nomenclature is used: Original amino acid, position, substituted amino acid. Accordingly, the substitution of threonine at position 226 with alanine is designated as “Thr226Ala” or “T226A”. Multiple mutations are separated by addition marks (“+”), e.g., “Gly205Arg+Ser411 Phe” or “G205R +S411 F”, representing substitutions at positions 205 and 411 of glycine (G) with arginine (R) and serine (S) with phenylalanine (F), respectively.

Multiple Substitutions. Variants comprising multiple substitutions are separated by addition marks (“+”), e.g., “Arg170Tyr+Gly195Glu” or “R170Y+G195E” representing a substitution of arginine and glycine at positions 170 and 195 with tyrosine and glutamic acid, respectively.

Different Substitutions. Where different substitutions can be introduced at a position, the different substitutions are separated by a comma, e.g., “Arg170Tyr,Glu” represents a substitution of arginine at position 170 with tyrosine or glutamic acid. Thus, “Tyr167Gly,Ala+Arg170Gly,Ala” designates the following variants: “Tyr167Gly+Arg170Gly”, “Tyr167Gly+Arg170Ala”, “Tyr167Ala+Arg170Gly”, and “Tyr167Ala+Arg170Ala”.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to isolated cutinase variants, comprising a substitution at one or more (e.g., several) positions corresponding to amino acid residue 36, 45, 52, 70, 120, 174 of the mature polypeptide of SEQ ID NO: 2, wherein the variant has cutinase activity.

Variants

The present invention relates to an isolated variant of a parent cutinase comprising a substitution at one or more positions corresponding to amino acid residue 31, 36, 45, 52, 70, 120, 143, 167, and 174 of the mature polypeptide of SEQ ID NO: 2 selected from the group consisting of: P31A,R,N,D,C,Q,E,G,H,I,L,K,M,F,S,T,W,Y,V; 136A,R,N,D,C,Q,E,G,H,L,K,M,F,P,S,T,W,Y,V; G45A,R,N,D,C,Q,E,H,I,L,K,M,F,P,S,T,W,Y,V; N52E; F70A,R,N,D,C,Q,E,G,H,I,L,K,M,P,S,T,W,Y,V; G120S; G143A,R,N,D,C,Q,E,H,I,L,K,M,F,P,S,T,W,Y,V; L167A; and L174N,Q, and which variant has cutinase activity.

In an embodiment, the variant has sequence identity of at least 60%, e.g., at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, but less than 100%, to the amino acid sequence of the parent cutinase.

In another embodiment, the variant has at least 70%, e.g., at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, such as at least 96%, at least 97%, at least 98%, or at least 99%, but less than 100%, sequence identity to the mature polypeptide of SEQ ID NO: 2.

In one aspect, the number of substitutions in the variants of the present invention is 1-20, e.g., 1-10 and 1-5, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 substitutions.

In another aspect, a variant comprises a substitution at one or more (e.g., several) positions corresponding to positions P31A,R,N,D,C,Q,E,G,H,I,L,K,M,F,S,T,W,Y,V; 136A,R,N,D,C,Q,E,G,H,L,K,M,F,P,S,T,W,Y,V; G45A,R,N,D,C,Q,E,H,I,L,K,M,F,P,S,T,W,Y,V; N52E; F70A,R,N,D,C,Q,E,G,H,I,L,K,M,P,S,T,W,Y,V; G120S; G143A,R,N,D,C,Q,E,H,I,L,K,

M,F,P,S,T,W,Y,V; L167A; and L174N,Q. Preferably the variant comprises a substitution at one, two, three, four, five, six, seven, eight, or nine positions corresponding to any of positions P31A,R,N,D,C,Q,E,G,H,I,L,K,M,F,S,T,W,Y,V; 136A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V; G45A,R,N,D,C,Q,E,H,I,L,K,M,F,P,S,T,W,Y,V; N52E; F70A,R,N,D,C,Q,E,G,H,I,L,K,M,P,S,T,W,Y,V; G120S; G143A,R,N,D,C,Q,E,H,I,L,K,M,F,P,S,T,W,Y,V; L167A; and L174N,Q.

In another aspect, the variant comprises or consists of a substitution at a position corresponding to position 31. In another aspect, the amino acid at a position corresponding to position 31 is substituted with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Ser, Thr, Trp, Tyr, or Val. In another aspect, the variant comprises or consists of the substitution P31A,R,N,D,C,Q,E,G,H,I,L,K,M,F,S,T,W,Y,V of the mature polypeptide of SEQ ID NO: 2.

In another aspect, the variant comprises or consists of a substitution at a position corresponding to position 36. In another aspect, the amino acid at a position corresponding to position 36 is substituted with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val.

Ser, Thr, Trp, Tyr, or Val. In another aspect, the variant comprises or consists of the substitution 136A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V of the mature polypeptide of SEQ ID NO: 2.

In another aspect, the variant comprises or consists of a substitution at a position corresponding to position 45. In another aspect, the amino acid at a position corresponding to position 45 is substituted with Ala, Arg, Asn, Asp, Cys, Gln, Glu, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val. In another aspect, the variant comprises or consists of the substitution G45A,R,N,D,C,Q,E,H,I,L,K,M,F,P,S,T,W,Y,V of the mature polypeptide of SEQ ID NO: 2.

In another aspect, the variant comprises or consists of a substitution at a position corresponding to position 52. In another aspect, the amino acid at a position corresponding to position 52 is substituted with Ala, Arg, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val, preferably with Glu. In another aspect, the variant comprises or consists of the substitution N52E of the mature polypeptide of SEQ ID NO: 2.

In another aspect, the variant comprises or consists of a substitution at a position corresponding to position 70. In another aspect, the amino acid at a position corresponding to position 70 is substituted with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Pro, Ser, Thr, Trp, Tyr, or Val. In another aspect, the variant comprises or consists of the substitution F70A,R,N,D,C,Q,E,G,H,I,L,K,M,P,S,T,W,Y,V of the mature polypeptide of SEQ ID NO: 2.

In another aspect, the variant comprises or consists of a substitution at a position corresponding to position 120. In another aspect, the amino acid at a position corresponding to position 120 is substituted with Ala, Arg, Asn, Asp, Cys, Gln, Glu, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val, preferably with Ser. In another aspect, the variant comprises or consists of the substitution G120S of the mature polypeptide of SEQ ID NO: 2.

In another aspect, the variant comprises or consists of a substitution at a position corresponding to position 143. In another aspect, the amino acid at a position corresponding to position 143 is substituted with Ala, Arg, Asn, Asp, Cys, Gln, Glu, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val. In another aspect, the variant comprises or consists of the substitution G143A,R,N,D,C,Q,E,H,I,L,K,M,F,P,S,T,W,Y,V of the mature polypeptide of SEQ ID NO: 2.

In another aspect, the variant comprises or consists of a substitution at a position corresponding to position 167. In

another aspect, the amino acid at a position corresponding to position 167 is substituted with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val, preferably with Ala. In another aspect, the variant comprises or consists of the substitution L167A of the mature polypeptide of SEQ ID NO: 2.

In another aspect, the variant comprises or consists of a substitution at a position corresponding to position 174. In another aspect, the amino acid at a position corresponding to position 174 is substituted with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val, preferably with Asn, or Gln. In another aspect, the variant comprises or consists of the substitution L174N,Q of the mature polypeptide of SEQ ID NO: 2.

In some embodiment the invention relates to the variant, which comprises or consists of at least one of the mutations/combination of mutations selected from: (a) E30Q; (b) P31V; (c) 136N; (d) 136Q; (e) 136S; (f) G45N; (g) R51P; (h) N52E; (i) F70A; (j) F70S; (k) G120S; (l) G143R; (m)

L167A; (n) L174N; (o) L174Q; (p) A14P/I169A; (q) A14P +N52E +I169A; or (r) G8D +A14P +R51P +E179Q.

The variants may further comprise one or more additional substitutions at one or more (e.g., several) other positions.

The amino acid changes may be of a minor nature, that is conservative amino acid substitutions or insertions that do not significantly affect the folding and/or activity of the protein; small deletions, typically of 1-30 amino acids; small amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue; a small linker peptide of up to 20-25 residues; or a small extension that facilitates purification by changing net charge or another function, such as a poly-histidine tract, an antigenic epitope or a binding domain.

Examples of conservative substitutions are within the groups of basic amino acids (arginine, lysine and histidine), acidic amino acids (glutamic acid and aspartic acid), polar amino acids (glutamine and asparagine), hydrophobic amino acids (leucine, isoleucine and valine), aromatic amino acids (phenylalanine, tryptophan and tyrosine), and small amino acids (glycine, alanine, serine, threonine and methionine). Amino acid substitutions that do not generally alter specific activity are known in the art and are described, for example, by H. Neurath and R.L. Hill, 1979, In, *The Proteins*, Academic Press, New York. Common substitutions are Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Nal, Ser/Gly, Tyr/Phe, Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile, Leu/Val, Ala/Glu, and Asp/Gly.

Alternatively, the amino acid changes are of such a nature that the physico-chemical properties of the polypeptides are altered. For example, amino acid changes may improve the thermal stability of the polypeptide, alter the substrate specificity, change the pH optimum, and the like.

The variants may also comprise a substitution at a position corresponding to positions: G8D, A14P, E30Q, E47R, E47K, R51P, I169A, I169V, or E179Q of the mature polypeptide of SEQ ID NO: 2.

Essential amino acids in a polypeptide can be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, 1989, *Science* 244: 1081-1085). In the latter technique, single alanine mutations are introduced at every residue in the molecule, and the resultant mutant molecules are tested for cutinase activity to identify amino acid residues that are critical to the activity of the molecule. See also, Hilton et al., 1996, *J. Biol. Chem.* 271: 4699-4708. The active site of the enzyme or other biological interaction can also be determined by physical analysis of structure, as

determined by such techniques as nuclear magnetic resonance, crystallography, electron diffraction, or photoaffinity labeling, in conjunction with mutation of putative contact site amino acids. See, for example, de Vos et al., 1992, *Science* 255: 306-312; Smith et al., 1992, *J. Mol. Biol.* 224: 899-904; Wlodaver et al., 1992, *FEBS Lett.* 309: 59-64. The identity of essential amino acids can also be inferred from an alignment with a related polypeptide.

The variants may consist of 100 to 194, 150 to 194, 100 to 150, or 150 to 194 amino acids.

In some embodiments the invention relates to a variant, wherein the cutinase activity of the variant relative to the cutinase activity of the parent is higher.

In some embodiments the invention relates to a variant, wherein the cutinase activity is measured on short-chained alkyl ester substrates, preferably wherein the substrate is selected from the group consisting of: methyl isobutyrate, methyl trimethylacetate, methyl crotonate, and methyl methacrylate.

In an embodiment, the variant has improved catalytic efficiency compared to the parent enzyme.

In an embodiment, the variant has improved catalytic rate compared to the parent enzyme. In an embodiment, the variant has improved specific activity compared to the parent enzyme.

In an embodiment, the variant has improved substrate binding compared to the parent enzyme.

In an embodiment, the variant has improved substrate cleavage compared to the parent enzyme.

In an embodiment, the variant has improved substrate specificity compared to the parent enzyme.

Parent Cutinases

The parent cutinase may be (a) a polypeptide having at least 70% sequence identity to the mature polypeptide of SEQ ID NO: 2; (b) a polypeptide encoded by a polynucleotide having at least 70% sequence identity to the mature polypeptide coding sequence of SEQ ID NO: 1.

In an aspect, the parent has a sequence identity to the mature polypeptide of SEQ ID NO: 2 of at least 70%, e.g., at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%, which have cutinase activity. In one aspect, the amino acid sequence of the parent differs by no more than 20 amino acids such as 1-10, 1-5, or, e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 from the mature polypeptide of SEQ ID NO: 2.

In another aspect, the parent comprises or consists of the amino acid sequence of SEQ ID NO: 2. In another aspect, the parent comprises or consists of the mature polypeptide of SEQ ID NO: 2. In another aspect, the parent comprises or consists of amino acids 1 to 194 of SEQ ID NO: 2.

In another aspect, the parent is a fragment of the mature polypeptide of SEQ ID NO: 2 containing at least 194 amino acid residues, e.g., at least 150 and at least 100 amino acid residues.

In another embodiment, the parent is an allelic variant of the mature polypeptide of SEQ ID NO: 2.

In another aspect, the parent is encoded by a polynucleotide that hybridizes under very low stringency conditions, low stringency conditions, medium stringency conditions, medium-high stringency conditions, high stringency conditions, or very high stringency conditions with (i) the mature polypeptide coding sequence of SEQ ID NO: 1, or (ii) the full-length complement of (i) (Sambrook et al., 1989, *Molecular Cloning, A Laboratory Manual*, 2d edition, Cold Spring Harbor, New York).

The polynucleotide of SEQ ID NO: 1 or a subsequence thereof, as well as the polypeptide of SEQ ID NO: 2 or a fragment thereof, may be used to design nucleic acid probes to identify and clone DNA encoding a parent from strains of different genera or species according to methods well known in the art. In particular, such probes can be used for hybridization with the genomic DNA or cDNA of a cell of interest, following standard Southern blotting procedures, in order to identify and isolate the corresponding gene therein. Such probes can be considerably shorter than the entire sequence, but should be at least 15, e.g., at least 25, at least 35, or at least 70 nucleotides in length. Preferably, the nucleic acid probe is at least 100 nucleotides in length, e.g., at least 200 nucleotides, at least 300 nucleotides, at least 400 nucleotides, at least 500 nucleotides, at least 600 nucleotides, at least 700 nucleotides, at least 800 nucleotides, or at least 900 nucleotides in length. Both DNA and RNA probes can be used. The probes are typically labeled for detecting the corresponding gene (for example, with ³²P, ³H, ³⁵S, biotin, or avidin). Such probes are encompassed by the present invention.

A genomic DNA or cDNA library prepared from such other strains may be screened for DNA that hybridizes with the probes described above and encodes a parent. Genomic or other DNA from such other strains may be separated by agarose or polyacrylamide gel electrophoresis, or other separation techniques. DNA from the libraries or the separated DNA may be transferred to and immobilized on nitrocellulose or other suitable carrier material. In order to identify a clone or DNA that hybridizes with SEQ ID NO: 1 or a subsequence thereof, the carrier material is used in a Southern blot.

For purposes of the present invention, hybridization indicates that the polynucleotide hybridizes to a labeled nucleic acid probe corresponding to (i) SEQ ID NO: 1; (ii) the mature polypeptide coding sequence of SEQ ID NO: 1; (iii) the full-length complement thereof; or (iv) a subsequence thereof; under very low to very high stringency conditions. Molecules to which the nucleic acid probe hybridizes under these conditions can be detected using, for example, X-ray film or any other detection means known in the art.

In one aspect, the nucleic acid probe is the mature polypeptide coding sequence of SEQ ID NO: 1. In another aspect, the nucleic acid probe is a polynucleotide that encodes the polypeptide of SEQ ID NO: 2; the mature polypeptide thereof; or a fragment thereof. In another aspect, the nucleic acid probe is SEQ ID NO: 1.

In another embodiment, the parent is encoded by a polynucleotide having a sequence identity to the mature polypeptide coding sequence of SEQ ID NO: 1 of at least 70%, e.g., at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%.

The polypeptide may be a hybrid polypeptide in which a region of one polypeptide is fused at the N-terminus or the C-terminus of a region of another polypeptide.

The parent may be a fusion polypeptide or cleavable fusion polypeptide in which another polypeptide is fused at the N-terminus or the C-terminus of the polypeptide of the present invention. A fusion polypeptide is produced by fusing a polynucleotide encoding another polypeptide to a polynucleotide of the present invention. Techniques for producing fusion polypeptides are known in the art, and include ligating the coding sequences encoding the polypeptides so that they are in frame and that expression of the fusion polypeptide is under control of the same promoter(s).

and terminator. Fusion polypeptides may also be constructed using intein technology in which fusion polypeptides are created post-translationally (Cooper et al., 1993, *EMBO J.* 12: 2575-2583; Dawson et al., 1994, *Science* 266: 776-779).

A fusion polypeptide can further comprise a cleavage site between the two polypeptides. Upon secretion of the fusion protein, the site is cleaved releasing the two polypeptides. Examples of cleavage sites include, but are not limited to, the sites disclosed in Martin et al., 2003, *J. Ind. Microbiol. Biotechnol.* 3: 568-576; Svetina et al., 2000, *J. Biotechnol.* 76: 245-251; Rasmussen-Wilson et al., 1997, *Appl. Environ. Microbiol.* 63: 3488-3493; Ward et al., 1995, *Biotechnology* 13: 498-503; and Contreras et al., 1991, *Biotechnology* 9: 378-381; Eaton et al., 1986, *Biochemistry* 25: 505-512; Collins-Racie et al., 1995, *Biotechnology* 13: 982-987; Carter et al., 1989, *Proteins: Structure, Function, and Genetics* 6: 240-248; and Stevens, 2003, *Drug Discovery World* 4: 35-48.

The parent may be obtained from microorganisms of any genus. For purposes of the present invention, the term "obtained from" as used herein in connection with a given source shall mean that the parent encoded by a polynucleotide is produced by the source or by a strain in which the polynucleotide from the source has been inserted. In one aspect, the parent is secreted extracellularly.

The parent may be a bacterial cutinase. For example, the parent may be a Gram-positive bacterial polypeptide such as a *Bacillus*, *Clostridium*, *Enterococcus*, *Geobacillus*, *Lactobacillus*, *Lactococcus*, *Oceanobacillus*, *Staphylococcus*, *Streptococcus*, or *Streptomyces* cutinase, or a Gram-negative bacterial polypeptide such as a *Campylobacter*, *E. coli*, *Flavobacterium*, *Fusobacterium*, *Helicobacter*, *Ilyobacter*, *Neisseria*, *Pseudomonas*, *Salmonella*, or *Ureaplasma* cutinase.

In one aspect, the parent is a *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus brevis*, *Bacillus circulans*, *Bacillus clausii*, *Bacillus coagulans*, *Bacillus firmus*, *Bacillus lautus*, *Bacillus lentus*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus pumilus*, *Bacillus stearothermophilus*, *Bacillus subtilis*, or *Bacillus thuringiensis* cutinase.

In another aspect, the parent is a *Streptococcus equisimilis*, *Streptococcus pyogenes*, *Streptococcus uberis*, or *Streptococcus equi* subsp. *Zooepidemicus* cutinase.

In another aspect, the parent is a *Streptomyces achromogenes*, *Streptomyces avermitilis*, *Streptomyces coelicolor*, *Streptomyces griseus*, or *Streptomyces lividans* cutinase.

The parent may be a fungal cutinase. For example, the parent may be a yeast cutinase such as a *Candida*, *Kluyveromyces*, *Pichia*, *Saccharomyces*, *Schizosaccharomyces*, or *Yarrowia* cutinase; or a filamentous fungal cutinase such as an *Acremonium*, *Agaricus*, *Altemaria*, *Aspergillus*, *Aureobasidium*, *Botryosphaeria*, *Ceriporiopsis*, *Chaetomidium*, *Chrysosporium*, *Claviceps*, *Cochliobolus*, *Coprinopsis*, *Coptotermes*, *Corynascus*, *Cryphonectria*, *Cryptococcus*, *Diplodia*, *Exidia*, *Filibasidium*, *Fusarium*, *Gibberella*, *Holomastigotoides*, *Humicola*, *Irpex*, *Lentinula*, *Leptosphaeria*, *Magnaporthe*, *Melanocarpus*, *Meripilus*, *Mucor*, *Myceliophthora*, *Neocallimastix*, *Neurospora*, *Paecilomyces*, *Penicillium*, *Phanerochaete*, *Piromyces*, *Poitrasia*, *Pseudoplectania*, *Pseudotrichonympha*, *Rhizomucor*, *Schizophyllum*, *Scytalidium*, *Talaromyces*, *Thermoascus*, *Thielavia*, *Tolypocladium*, *Trichoderma*, *Trichophaea*, *Verticillium*, *Volvariella*, or *Xylaria* cutinase.

In another aspect, the parent is a *Saccharomyces carlsbergensis*, *Saccharomyces cerevisiae*, *Saccharomyces dia-*

staticus, *Saccharomyces douglasii*, *Saccharomyces kluyveri*, *Saccharomyces norbensis*, or *Saccharomyces oviformis* cutinase.

In another aspect, the parent is an *Acremonium cellulolyticus*, *Aspergillus aculeatus*, *Aspergillus awamori*, *Aspergillus foetidus*, *Aspergillus fumigatus*, *Aspergillus japonicus*, *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus oryzae*, *Chrysosporium inops*, *Chrysosporium keratinophilum*, *Chrysosporium lucknowense*, *Chrysosporium merdarium*, *Chrysosporium pannicola*, *Chrysosporium queenslandicum*, *Chrysosporium tropicum*, *Chrysosporium zonatum*, *Fusarium bactridioides*, *Fusarium cerealis*, *Fusarium crookwellense*, *Fusarium culmorum*, *Fusarium graminearum*, *Fusarium graminum*, *Fusarium heterosporum*, *Fusarium negundi*, *Fusarium oxysporum*, *Fusarium reticulatum*, *Fusarium roseum*, *Fusarium sambucinum*, *Fusarium sarcochromum*, *Fusarium sporotrichioides*, *Fusarium sulphureum*, *Fusarium torulosum*, *Fusarium trichothecioides*, *Fusarium venenatum*, *Humicola grisea*, *Humicola insolens*, *Humicola lanuginosa*, *Irpex lacteus*, *Mucor miehei*, *Myceliophthora thermophila*, *Neurospora crassa*, *Penicillium funiculosum*, *Penicillium purpurogenum*, *Phanerochaete chrysosporium*, *Thielavia achromatica*, *Thielavia albomyces*, *Thielavia albopilosa*, *Thielavia australeinsis*, *Thielavia fimeti*, *Thielavia microspora*, *Thielavia ovispora*, *Thielavia peruviana*, *Thielavia setosa*, *Thielavia spededonium*, *Thielavia subthermophila*, *Thielavia terrestris*, *Trichoderma harzianum*, *Trichoderma kongii*, *Trichoderma longibrachiatum*, *Trichoderma reesei*, or *Trichoderma viride* cutinase.

In another aspect, the parent is a *Humicola insolens* cutinase e.g., the cutinase of SEQ ID NO: 2 or the mature polypeptide thereof.

It will be understood that for the aforementioned species, the invention encompasses both the perfect and imperfect states, and other taxonomic equivalents, e.g., anamorphs, regardless of the species name by which they are known. Those skilled in the art will readily recognize the identity of appropriate equivalents.

Strains of these species are readily accessible to the public in a number of culture collections, such as the American Type Culture Collection (ATCC), Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Centraalbureau Voor Schimmelcultures (CBS), and Agricultural Research Service Patent Culture Collection, Northern Regional Research Center (NRRRL).

The parent may be identified and obtained from other sources including microorganisms isolated from nature (e.g., soil, composts, water, etc.) or DNA samples obtained directly from natural materials (e.g., soil, composts, water, etc.) using the above-mentioned probes. Techniques for isolating microorganisms and DNA directly from natural habitats are well known in the art. A polynucleotide encoding a parent may then be obtained by similarly screening a genomic DNA or cDNA library of another microorganism or mixed DNA sample. Once a polynucleotide encoding a parent has been detected with the probe(s), the polynucleotide can be isolated or cloned by utilizing techniques that are known to those of ordinary skill in the art (see, e.g., Sambrook et al., 1989, supra).

Preparation of Variants

The present invention also relates to methods for obtaining a variant having cutinase activity, comprising: (a) introducing into a parent cutinase a substitution at one or more (e.g., several) positions corresponding to positions P31A,R, N,D,C,Q,E,G,H,I,L,K,M,F,S,T,W,Y,V; I36A,R,N,D,C,Q,E, G,H,L,K,M,F,P,S,T,W,Y,V; G45A,R,N,D,C,Q,E,H,I,L,K,M,

F,P,S,T,W,Y,V; N52E; F70A,R,N,D,C,Q,E,G,H,I,L,K,M,P,S,T,W,Y,V; G120S; G143A,R,N,D,C,Q,E,H,I,L,K,M,F,P,S,T,W,Y,V; L167A; and L174N,Q of the mature polypeptide of SEQ ID NO: 2, wherein the variant has cutinase activity; and (b) recovering the variant.

The variants can be prepared using any mutagenesis procedure known in the art, such as site-directed mutagenesis, synthetic gene construction, semi-synthetic gene construction, random mutagenesis, shuffling, etc.

Site-directed mutagenesis is a technique in which one or more (e.g., several) mutations are introduced at one or more defined sites in a polynucleotide encoding the parent.

Site-directed mutagenesis can be accomplished in vitro by PCR involving the use of oligonucleotide primers containing the desired mutation. Site-directed mutagenesis can also be performed in vitro by cassette mutagenesis involving the cleavage by a restriction enzyme at a site in the plasmid comprising a polynucleotide encoding the parent and subsequent ligation of an oligonucleotide containing the mutation in the polynucleotide. Usually the restriction enzyme that digests the plasmid and the oligonucleotide is the same, permitting sticky ends of the plasmid and the insert to ligate to one another. See, e.g., Scherer and Davis, 1979, *Proc. Natl. Acad. Sci. USA* 76: 4949-4955; and Barton et al., 1990, *Nucleic Acids Res.* 18: 7349-4966.

Site-directed mutagenesis can also be accomplished in vivo by methods known in the art. See, e.g., US2004/0171154; Storici et al., 2001, *Nature Biotechnol.* 19: 773-776; Kren et al., 1998, *Nat. Med.* 4: 285-290; and Calissano and Macino, 1996, *Fungal Genet. Newslett.* 43: 15-16.

Any site-directed mutagenesis procedure can be used in the present invention. There are many commercial kits available that can be used to prepare variants.

Synthetic gene construction entails in vitro synthesis of a designed polynucleotide molecule to encode a polypeptide of interest. Gene synthesis can be performed utilizing a number of techniques, such as the multiplex microchip-based technology described by Tian et al. (2004, *Nature* 432: 1050-1054) and similar technologies wherein oligonucleotides are synthesized and assembled upon photo-programmable microfluidic chips.

Single or multiple amino acid substitutions, deletions, and/or insertions can be made and tested using known methods of mutagenesis, recombination, and/or shuffling, followed by a relevant screening procedure, such as those disclosed by Reidhaar-Olson and Sauer, 1988, *Science* 241: 53-57; Bowie and Sauer, 1989, *Proc. Natl. Acad. Sci. USA* 86: 2152-2156; WO95/17413; or WO95/22625. Other methods that can be used include error-prone PCR, phage display (e.g., Lowman et al., 1991, *Biochemistry* 30: 10832-10837; US5223409; WO92/06204) and region-directed mutagenesis (Derbyshire et al., 1986, *Gene* 46: 145; Ner et al., 1988, *DNA* 7: 127).

Mutagenesis/shuffling methods can be combined with high-throughput, automated screening methods to detect activity of cloned, mutagenized polypeptides expressed by host cells (Ness et al., 1999, *Nature Biotechnology* 17: 893-896). Mutagenized DNA molecules that encode active polypeptides can be recovered from the host cells and rapidly sequenced using standard methods in the art. These methods allow the rapid determination of the importance of individual amino acid residues in a polypeptide.

Semi-synthetic gene construction is accomplished by combining aspects of synthetic gene construction, and/or site-directed mutagenesis, and/or random mutagenesis, and/or shuffling. Semi-synthetic construction is typified by a process utilizing polynucleotide fragments that are synthe-

sized, in combination with PCR techniques. Defined regions of genes may thus be synthesized de novo, while other regions may be amplified using site-specific mutagenic primers, while yet other regions may be subjected to error-prone PCR or non-error prone PCR amplification. Polynucleotide subsequences may then be shuffled.

Polynucleotides

The present invention also relates to isolated polynucleotides encoding a variant of the present invention.

Nucleic Acid Constructs

The present invention also relates to nucleic acid constructs comprising a polynucleotide encoding a variant of the present invention operably linked to one or more control sequences that direct the expression of the coding sequence in a suitable host cell under conditions compatible with the control sequences.

The polynucleotide may be manipulated in a variety of ways to provide for expression of a variant. Manipulation of the polynucleotide prior to its insertion into a vector may be desirable or necessary depending on the expression vector. The techniques for modifying polynucleotides utilizing recombinant DNA methods are well known in the art. The control sequence may be a promoter, a polynucleotide which is recognized by a host cell for expression of the polynucleotide. The promoter contains transcriptional control sequences that mediate the expression of the variant. The promoter may be any polynucleotide that shows transcriptional activity in the host cell including mutant, truncated, and hybrid promoters, and may be obtained from genes encoding extracellular or intracellular polypeptides either homologous or heterologous to the host cell.

Examples of suitable promoters for directing transcription of the nucleic acid constructs of the present invention in a bacterial host cell are the promoters obtained from the *Bacillus amyloliquefaciens* alpha-amylase gene (amyQ), *Bacillus licheniformis* alpha-amylase gene (amyL), *Bacillus licheniformis* penicillinase gene (penP), *Bacillus stearothermophilus* maltogenic amylase gene (amyM), *Bacillus subtilis* levansucrase gene (sacB), *Bacillus subtilis* xylA and xylB genes, *Bacillus thuringiensis* cryIIIA gene (Agaïsse and Lereclus, 1994, *Molecular Microbiology* 13: 97-107), *E. coli* lac operon, *E. coli* trc promoter (Egon et al., 1988, *Gene* 69: 301-315), *Streptomyces coelicolor* agarase gene (dagA), and prokaryotic beta-lactamase gene (Villa-Kamaroff et al., 1978, *Proc. Natl. Acad. Sci. USA* 75: 3727-3731), as well as the tac promoter (DeBoer et al., 1983, *Proc. Natl. Acad. Sci. USA* 80: 21-25). Further promoters are described in "Useful proteins from recombinant bacteria" in Gilbert et al., 1980, *Scientific American* 242: 74-94; and in Sambrook et al., 1989, supra. Examples of tandem promoters are disclosed in WO99/43835.

Examples of suitable promoters for directing transcription of the nucleic acid constructs of the present invention in a filamentous fungal host cell are promoters obtained from the genes for *Aspergillus nidulans* acetamidase, *Aspergillus niger* neutral alpha-amylase, *Aspergillus niger* acid stable alpha-amylase, *Aspergillus niger* or *Aspergillus awamori* glucoamylase (glaA), *Aspergillus oryzae* TAKA amylase, *Aspergillus oryzae* alkaline protease, *Aspergillus oryzae* triose phosphate isomerase, *Fusarium oxysporum* trypsin-like protease (WO96/00787), *Fusarium venenatum* amyloglucosidase (WO00/56900), *Fusarium venenatum* Daria (WO00/56900), *Fusarium venenatum* Quinn (WO00/56900), *Rhizomucor miehei* lipase, *Rhizomucor miehei* aspartic proteinase, *Trichoderma reesei* beta-glucosidase, *Trichoderma reesei* cellobiohydrolase I, *Trichoderma reesei* cellobiohydrolase II, *Trichoderma reesei* endoglucanase I,

Trichoderma reesei endoglucanase II, *Trichoderma reesei* endoglucanase III, *Trichoderma reesei* endoglucanase IV, *Trichoderma reesei* endoglucanase V, *Trichoderma reesei* xylanase I, *Trichoderma reesei* xylanase II, *Trichoderma reesei* beta-xylosidase, as well as the NA2-tpi promoter (a modified promoter from an *Aspergillus neutral* alpha-amylase gene in which the untranslated leader has been replaced by an untranslated leader from an *Aspergillus* triose phosphate isomerase gene; non-limiting examples include modified promoters from an *Aspergillus niger* neutral alpha-amylase gene in which the untranslated leader has been replaced by an untranslated leader from an *Aspergillus nidulans* or *Aspergillus oryzae* triose phosphate isomerase gene); and mutant, truncated, and hybrid promoters thereof.

In a yeast host, useful promoters are obtained from the genes for *Saccharomyces cerevisiae* enolase (ENO-1), *Saccharomyces cerevisiae* galactokinase (GAL1), *Saccharomyces cerevisiae* alcohol dehydrogenase/glyceraldehyde-3-phosphate dehydrogenase (ADH1, ADH2/GAP), *Saccharomyces cerevisiae* triose phosphate isomerase (TPI), *Saccharomyces cerevisiae* metallothionein (CUP1), and *Saccharomyces cerevisiae* 3-phosphoglycerate kinase. Other useful promoters for yeast host cells are described by Romanos et al., 1992, *Yeast* 8: 423-488.

The control sequence may also be a transcription terminator, which is recognized by a host cell to terminate transcription. The terminator sequence is operably linked to the 3'-terminus of the polynucleotide encoding the variant. Any terminator that is functional in the host cell may be used.

Preferred terminators for bacterial host cells are obtained from the genes for *Bacillus clausii* alkaline protease (aprH), *Bacillus licheniformis* alpha-amylase (amyL), and *Escherichia coli* ribosomal RNA (rrnB).

Preferred terminators for filamentous fungal host cells are obtained from the genes for *Aspergillus nidulans* anthranilate synthase, *Aspergillus niger* glucoamylase, *Aspergillus niger* alpha-glucosidase, *Aspergillus oryzae* TAKA amylase, and *Fusarium oxysporum* trypsin-like protease.

Preferred terminators for yeast host cells are obtained from the genes for *Saccharomyces cerevisiae* enolase, *Saccharomyces cerevisiae* cytochrome C (CYC1), and *Saccharomyces cerevisiae* glyceraldehyde-3-phosphate dehydrogenase. Other useful terminators for yeast host cells are described by Romanos et al., 1992, supra.

The control sequence may also be an mRNA stabilizer region downstream of a promoter and upstream of the coding sequence of a gene which increases expression of the gene.

Examples of suitable mRNA stabilizer regions are obtained from a *Bacillus thuringiensis* cryIIIA gene (WO94/25612) and a *Bacillus subtilis* SP82 gene (Hue et al., 1995, *Journal of Bacteriology* 177: 3465-3471).

The control sequence may also be a leader, a nontranslated region of an mRNA that is important for translation by the host cell. The leader sequence is operably linked to the 5'-terminus of the polynucleotide encoding the variant. Any leader that is functional in the host cell may be used.

Preferred leaders for filamentous fungal host cells are obtained from the genes for *Aspergillus oryzae* TAKA amylase and *Aspergillus nidulans* triose phosphate isomerase.

Suitable leaders for yeast host cells are obtained from the genes for *Saccharomyces cerevisiae* enolase (ENO-1), *Saccharomyces cerevisiae* 3-phosphoglycerate kinase, *Saccharomyces cerevisiae* alpha-factor, and *Saccharomyces cerevi-*

siae alcohol dehydrogenase/glyceraldehyde-3-phosphate dehydrogenase (ADH2/GAP).

The control sequence may also be a polyadenylation sequence, a sequence operably linked to the 3'-terminus of the variant-encoding sequence and, when transcribed, is recognized by the host cell as a signal to add polyadenosine residues to transcribed mRNA. Any polyadenylation sequence that is functional in the host cell may be used.

Preferred polyadenylation sequences for filamentous fungal host cells are obtained from the genes for *Aspergillus nidulans* anthranilate synthase, *Aspergillus niger* glucoamylase, *Aspergillus niger* alpha-glucosidase, *Aspergillus oryzae* TAKA amylase, and *Fusarium oxysporum* trypsin-like protease.

Useful polyadenylation sequences for yeast host cells are described by Guo and Sherman, 1995, *Mol. Cellular Biol.* 15: 5983-5990.

The control sequence may also be a signal peptide coding region that encodes a signal peptide linked to the N-terminus of a variant and directs the variant into the cell's secretory pathway. The 5'-end of the coding sequence of the polynucleotide may inherently contain a signal peptide coding sequence naturally linked in translation reading frame with the segment of the coding sequence that encodes the variant.

Alternatively, the 5'-end of the coding sequence may contain a signal peptide coding sequence that is foreign to the coding sequence. A foreign signal peptide coding sequence may be required where the coding sequence does not naturally contain a signal peptide coding sequence. Alternatively, a foreign signal peptide coding sequence may simply replace the natural signal peptide coding sequence in order to enhance secretion of the variant. However, any signal peptide coding sequence that directs the expressed variant into the secretory pathway of a host cell may be used.

Effective signal peptide coding sequences for bacterial host cells are the signal peptide coding sequences obtained from the genes for *Bacillus* NCIB 11837 maltogenic amylase, *Bacillus licheniformis* subtilisin, *Bacillus licheniformis* beta-lactamase, *Bacillus stearothermophilus* alpha-amylase, *Bacillus stearothermophilus* neutral proteases (nprT, nprS, nprM), and *Bacillus subtilis* prsA. Further signal peptides are described by Simonen and Palva, 1993, *Microbiological Reviews* 57: 109-137.

Effective signal peptide coding sequences for filamentous fungal host cells are the signal peptide coding sequences obtained from the genes for *Aspergillus niger* neutral amylase, *Aspergillus niger* glucoamylase, *Aspergillus oryzae* TAKA amylase, *Humicola insolens* cellulase, *Humicola insolens* endoglucanase V, *Humicola lanuginosa* lipase, and *Rhizomucor miehei* aspartic proteinase.

Useful signal peptides for yeast host cells are obtained from the genes for *Saccharomyces cerevisiae* alpha-factor and *Saccharomyces cerevisiae* invertase. Other useful signal peptide coding sequences are described by Romanos et al., 1992, supra.

The control sequence may also be a propeptide coding sequence that encodes a propeptide positioned at the N-terminus of a variant. The resultant polypeptide is known as a proenzyme or propolypeptide (or a zymogen in some cases). A propolypeptide is generally inactive and can be converted to an active polypeptide by catalytic or autocatalytic cleavage of the propeptide from the propolypeptide. The propeptide coding sequence may be obtained from the genes for *Bacillus subtilis* alkaline protease (aprE), *Bacillus subtilis* neutral protease (nprT), *Myceliophthora thermophila* laccase (WO95/33836), *Rhizomucor miehei* aspartic proteinase, and *Saccharomyces cerevisiae* alpha-factor.

Where both signal peptide and propeptide sequences are present, the propeptide sequence is positioned next to the N-terminus of the variant and the signal peptide sequence is positioned next to the N-terminus of the propeptide sequence.

It may also be desirable to add regulatory sequences that regulate expression of the variant relative to the growth of the host cell. Examples of regulatory systems are those that cause expression of the gene to be turned on or off in response to a chemical or physical stimulus, including the presence of a regulatory compound. Regulatory systems in prokaryotic systems include the lac, tac, and trp operator systems. In yeast, the ADH2 system or GAL1 system may be used. In filamentous fungi, the *Aspergillus niger* glucoamylase promoter, *Aspergillus oryzae* TAKA alpha-amylase promoter, and *Aspergillus oryzae* glucoamylase promoter may be used. Other examples of regulatory sequences are those that allow for gene amplification. In eukaryotic systems, these regulatory sequences include the dihydrofolate reductase gene that is amplified in the presence of methotrexate, and the metallothionein genes that are amplified with heavy metals. In these cases, the polynucleotide encoding the variant would be operably linked with the regulatory sequence.

Expression Vectors

The present invention also relates to recombinant expression vectors comprising a polynucleotide encoding a variant of the present invention, a promoter, and transcriptional and translational stop signals. The various nucleotide and control sequences may be joined together to produce a recombinant expression vector that may include one or more convenient restriction sites to allow for insertion or substitution of the polynucleotide encoding the variant at such sites. Alternatively, the polynucleotide may be expressed by inserting the polynucleotide or a nucleic acid construct comprising the polynucleotide into an appropriate vector for expression. In creating the expression vector, the coding sequence is located in the vector so that the coding sequence is operably linked with the appropriate control sequences for expression.

The recombinant expression vector may be any vector (e.g., a plasmid or virus) that can be conveniently subjected to recombinant DNA procedures and can bring about expression of the polynucleotide. The choice of the vector will typically depend on the compatibility of the vector with the host cell into which the vector is to be introduced. The vector may be a linear or closed circular plasmid.

The vector may be an autonomously replicating vector, i.e., a vector that exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g., a plasmid, an extrachromosomal element, a minichromosome, or an artificial chromosome. The vector may contain any means for assuring self-replication. Alternatively, the vector may be one that, when introduced into the host cell, is integrated into the genome and replicated together with the chromosome(s) into which it has been integrated. Furthermore, a single vector or plasmid or two or more vectors or plasmids that together contain the total DNA to be introduced into the genome of the host cell, or a transposon, may be used.

The vector preferably contains one or more selectable markers that permit easy selection of transformed, transfected, transduced, or the like cells. A selectable marker is a gene the product of which provides for biocide or viral resistance, resistance to heavy metals, prototrophy to auxotrophs, and the like.

Examples of bacterial selectable markers are *Bacillus licheniformis* or *Bacillus subtilis* dal genes, or markers that confer antibiotic resistance such as ampicillin, chloramphenicol, kanamycin, neomycin, spectinomycin or tetracycline resistance. Suitable markers for yeast host cells include, but are not limited to, ADE2, HIS3, LEU2, LYS2, MET3, TRP1, and URA3. Selectable markers for use in a filamentous fungal host cell include, but are not limited to, amdS (acetamidase), argB (ornithine carbamoyltransferase), bar (phosphinothricin acetyltransferase), hph (hygromycin phosphotransferase), niaD (nitrate reductase), pyrG (orotidine-5'-phosphate decarboxylase), sC (sulfate adenylyltransferase), and trpC (anthranilate synthase), as well as equivalents thereof. Preferred for use in an *Aspergillus* cell are *Aspergillus nidulans* or *Aspergillus oryzae* amdS and pyrG genes and a *Streptomyces hygrosopicus* bar gene.

The vector preferably contains an element(s) that permits integration of the vector into the host cell's genome or autonomous replication of the vector in the cell independent of the genome.

For integration into the host cell genome, the vector may rely on the polynucleotide's sequence encoding the variant or any other element of the vector for integration into the genome by homologous or non-homologous recombination. Alternatively, the vector may contain additional polynucleotides for directing integration by homologous recombination into the genome of the host cell at a precise location(s) in the chromosome(s). To increase the likelihood of integration at a precise location, the integrational elements should contain a sufficient number of nucleic acids, such as 100 to 10,000 base pairs, 400 to 10,000 base pairs, and 800 to 10,000 base pairs, which have a high degree of sequence identity to the corresponding target sequence to enhance the probability of homologous recombination. The integrational elements may be any sequence that is homologous with the target sequence in the genome of the host cell. Furthermore, the integrational elements may be non-encoding or encoding polynucleotides. On the other hand, the vector may be integrated into the genome of the host cell by non-homologous recombination.

For autonomous replication, the vector may further comprise an origin of replication enabling the vector to replicate autonomously in the host cell in question. The origin of replication may be any plasmid replicator mediating autonomous replication that functions in a cell. The term "origin of replication" or "plasmid replicator" means a polynucleotide that enables a plasmid or vector to replicate in vivo.

Examples of bacterial origins of replication are the origins of replication of plasmids pBR322, pUC19, pACYC177, and pACYC184 permitting replication in *E. coli*, and pUB110, pE194, pTA1060, and pAMβ1 permitting replication in *Bacillus*.

Examples of origins of replication for use in a yeast host cell are the 2 micron origin of replication, ARS1, ARS4, the combination of ARS1 and CEN3, and the combination of ARS4 and CEN6.

Examples of origins of replication useful in a filamentous fungal cell are AMA1 and ANSI (Gems et al., 1991, *Gene* 98: 61-67; Cullen et al., 1987, *Nucleic Acids Res.* 15: 9163-9175; WO00/24883). Isolation of the AMA1 gene and construction of plasmids or vectors comprising the gene can be accomplished according to the methods disclosed in WO00/24883.

More than one copy of a polynucleotide of the present invention may be inserted into a host cell to increase production of a variant. An increase in the copy number of the polynucleotide can be obtained by integrating at least

one additional copy of the sequence into the host cell genome or by including an amplifiable selectable marker gene with the polynucleotide where cells containing amplified copies of the selectable marker gene, and thereby additional copies of the polynucleotide, can be selected for by cultivating the cells in the presence of the appropriate selectable agent.

The procedures used to ligate the elements described above to construct the recombinant expression vectors of the present invention are well known to one skilled in the art (see, e.g., Sambrook et al., 1989, supra).

Host Cells

The present invention also relates to recombinant host cells, comprising a polynucleotide encoding a variant of the present invention operably linked to one or more control sequences that direct the production of a variant of the present invention. A construct or vector comprising a polynucleotide is introduced into a host cell so that the construct or vector is maintained as a chromosomal integrant or as a self-replicating extra-chromosomal vector as described earlier. The term "host cell" encompasses any progeny of a parent cell that is not identical to the parent cell due to mutations that occur during replication. The choice of a host cell will to a large extent depend upon the gene encoding the variant and its source.

The host cell may be any cell useful in the recombinant production of a variant, e.g., a prokaryote or a eukaryote.

The prokaryotic host cell may be any Gram-positive or Gram-negative bacterium. Gram-positive bacteria include, but are not limited to, *Bacillus*, *Clostridium*, *Enterococcus*, *Geobacillus*, *Lactobacillus*, *Lactococcus*, *Oceanobacillus*, *Staphylococcus*, *Streptococcus*, and *Streptomyces*. Gram-negative bacteria include, but are not limited to, *Campylobacter*, *E. coli*, *Flavobacterium*, *Fusobacterium*, *Helicobacter*, *Ilyobacter*, *Neisseria*, *Pseudomonas*, *Salmonella*, and *Ureaplasma*.

The bacterial host cell may be any *Bacillus* cell including, but not limited to, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus brevis*, *Bacillus circulans*, *Bacillus clausii*, *Bacillus coagulans*, *Bacillus firmus*, *Bacillus lautus*, *Bacillus lentus*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus pumilus*, *Bacillus stearothermophilus*, *Bacillus subtilis*, and *Bacillus thuringiensis* cells.

The bacterial host cell may also be any *Streptococcus* cell including, but not limited to, *Streptococcus equisimilis*, *Streptococcus pyogenes*, *Streptococcus uberis*, and *Streptococcus equi* subsp. *Zooepidemicus* cells.

The bacterial host cell may also be any *Streptomyces* cell, including, but not limited to, *Streptomyces achromogenes*, *Streptomyces avermitilis*, *Streptomyces coelicolor*, *Streptomyces griseus*, and *Streptomyces lividans* cells.

The introduction of DNA into a *Bacillus* cell may be effected by protoplast transformation (see, e.g., Chang and Cohen, 1979, *Mol. Gen. Genet.* 168: 111-115), competent cell transformation (see, e.g., Young and Spizizen, 1961, *J. Bacteriol.* 81: 823-829, or Dubnau and Davidoff-Abelson, 1971, *J. Mol. Biol.* 56: 209-221), electroporation (see, e.g., Shigekawa and Dower, 1988, *Biotechniques* 6: 742-751), or conjugation (see, e.g., Koehler and Thorne, 1987, *J. Bacteriol.* 169: 5271-5278). The introduction of DNA into an *E. coli* cell may be effected by protoplast transformation (see, e.g., Hanahan, 1983, *J. Mol. Biol.* 166: 557-580) or electroporation (see, e.g., Dower et al., 1988, *Nucleic Acids Res.* 16: 6127-6145). The introduction of DNA into a *Streptomyces* cell may be effected by protoplast transformation, electroporation (e.g., Gong et al., 2004, *Folia Microbiol. (Praha)* 49: 399-405), conjugation (see, e.g., Mazodier et

al., 1989, *J. Bacteriol.* 171: 3583-3585), or transduction (see, e.g., Burke et al., 2001, *Proc. Natl. Acad. Sci. USA* 98: 6289-6294). The introduction of DNA into a *Pseudomonas* cell may be effected by electroporation (see, e.g., Choi et al., 2006, *J. Microbiol. Methods* 64: 391-397), or conjugation (e.g., Pinedo and Smets, 2005, *Appl. Environ. Microbiol.* 71: 51-57). The introduction of DNA into a *Streptococcus* cell may be effected by natural competence (e.g., Perry and Kuramitsu, 1981, *Infect. Immun.* 32: 1295-1297), protoplast transformation (e.g., Catt and Jollick, 1991, *Microbios* 68: 189-207), electroporation (see, e.g., Buckley et al., 1999, *Appl. Environ. Microbiol.* 65: 3800-3804) or conjugation (see, e.g., Clewell, 1981, *Microbiol. Rev.* 45: 409-436). However, any method known in the art for introducing DNA into a host cell can be used.

The host cell may also be a eukaryote, such as a mammalian, insect, plant, or fungal cell.

The host cell may be a fungal cell. "Fungi" as used herein includes the phyla Ascomycota, Basidiomycota, Chytridiomycota, and Zygomycota as well as the Oomycota and all mitospore fungi (as defined by Hawksworth et al., *In, Ainsworth and Bisby's Dictionary of The Fungi*, 8th edition, 1995, CAB International, University Press, Cambridge, UK).

The fungal host cell may be a yeast cell. "Yeast" as used herein includes ascosporeogenous yeast (Endomycetales), basidiosporeogenous yeast, and yeast belonging to the Fungi Imperfecti (Blastomycetes). Since the classification of yeast may change in the future, for the purposes of this invention, yeast shall be defined as described in *Biology and Activities of Yeast* (Skinner, Passmore, and Davenport, editors, *Soc. App. Bacteriol. Symposium Series No. 9*, 1980).

The yeast host cell may be a *Candida*, *Hansenula*, *Kluyveromyces*, *Pichia*, *Saccharomyces*, *Schizosaccharomyces*, or *Yarrowia* cell such as a *Kluyveromyces lactis*, *Saccharomyces carlsbergensis*, *Saccharomyces cerevisiae*, *Saccharomyces diastaticus*, *Saccharomyces douglasii*, *Saccharomyces kluyveri*, *Saccharomyces norbensis*, *Saccharomyces oviformis*, or *Yarrowia lipolytica* cell.

The fungal host cell may be a filamentous fungal cell. "Filamentous fungi" include all filamentous forms of the subdivision Eumycota and Oomycota (as defined by Hawksworth et al., 1995, supra). The filamentous fungi are generally characterized by a mycelial wall composed of chitin, cellulose, glucan, chitosan, mannan, and other complex polysaccharides. Vegetative growth is by hyphal elongation and carbon catabolism is obligately aerobic. In contrast, vegetative growth by yeasts such as *Saccharomyces cerevisiae* is by budding of a unicellular thallus and carbon catabolism may be fermentative.

The filamentous fungal host cell may be an *Acremonium*, *Aspergillus*, *Aureobasidium*, *Bjerkandera*, *Ceriporiopsis*, *Chrysosporium*, *Coprinus*, *Coriolus*, *Cryptococcus*, *Filibasidium*, *Fusarium*, *Humicola*, *Magnaporthe*, *Mucor*, *Myceliophthora*, *Neocallimastix*, *Neurospora*, *Paecilomyces*, *Penicillium*, *Phanerochaete*, *Phlebia*, *Piromyces*, *Pleurotus*, *Schizophyllum*, *Talaromyces*, *Thermoascus*, *Thielavia*, *Tolypodadium*, *Trametes*, or *Trichoderma* cell.

For example, the filamentous fungal host cell may be an *Aspergillus awamori*, *Aspergillus foetidus*, *Aspergillus fumigatus*, *Aspergillus japonicus*, *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus oryzae*, *Bjerkandera adusta*, *Ceriporiopsis aneirina*, *Ceriporiopsis caregiea*, *Ceriporiopsis gilvescens*, *Ceriporiopsis pannocinta*, *Ceriporiopsis rivulosa*, *Ceriporiopsis subrufa*, *Ceriporiopsis subvermisporea*, *Chrysosporium inops*, *Chrysosporium keratinophilum*, *Chrysosporium lucknowense*, *Chrysosporium mer-*

darium, *Chrysosporium pannicola*, *Chrysosporium queenslandicum*, *Chrysosporium tropicum*, *Chrysosporium zonatum*, *Coprinus cinereus*, *Coriolus hirsutus*, *Fusarium bactridioides*, *Fusarium cerealis*, *Fusarium crookwellense*, *Fusarium culmorum*, *Fusarium graminearum*, *Fusarium graminum*, *Fusarium heterosporum*, *Fusarium negundi*, *Fusarium oxysporum*, *Fusarium reticulatum*, *Fusarium roseum*, *Fusarium sambucinum*, *Fusarium sarcochroum*, *Fusarium sporotrichioides*, *Fusarium sulphureum*, *Fusarium torulosum*, *Fusarium trichothecioides*, *Fusarium venenatum*, *Humicola insolens*, *Humicola lanuginosa*, *Mucor miehei*, *Myceliophthora thermophila*, *Neurospora crassa*, *Penicillium purpurogenum*, *Phanerochaete chrysosporium*, *Phlebia radiata*, *Pleurotus eryngii*, *Thielavia terrestris*, *Trametes villosa*, *Trametes versicolor*, *Trichoderma harzianum*, *Trichoderma koningii*, *Trichoderma longibrachiatum*, *Trichoderma reesei*, or *Trichoderma viride* cell.

Fungal cells may be transformed by a process involving protoplast formation, transformation of the protoplasts, and regeneration of the cell wall in a manner known per se. Suitable procedures for transformation of *Aspergillus* and *Trichoderma* host cells are described in EP238023, Yelton et al., 1984, *Proc. Natl. Acad. Sci. USA* 81: 1470-1474, and Christensen et al., 1988, *Bio/Technology* 6: 1419-1422. Suitable methods for transforming *Fusarium* species are described by Malardier et al., 1989, *Gene* 78: 147-156, and WO96/00787. Yeast may be transformed using the procedures described by Becker and Guarente, In Abelson, J. N. and Simon, M. I., editors, *Guide to Yeast Genetics and Molecular Biology, Methods in Enzymology*, Volume 194, pp 182-187, Academic Press, Inc., New York; Ito et al., 1983, *J. Bacteriol.* 153: 163; and Hinnen et al., 1978, *Proc. Natl. Acad. Sci. USA* 75: 1920.

Methods of Production

The present invention also relates to methods of producing a variant, comprising: (a) cultivating a host cell of the present invention under conditions suitable for expression of the variant; and (b) recovering the variant.

The host cells are cultivated in a nutrient medium suitable for production of the variant using methods known in the art. For example, the cell may be cultivated by shake flask cultivation, or small-scale or large-scale fermentation (including continuous, batch, fed-batch, or solid state fermentations) in laboratory or industrial fermentors performed in a suitable medium and under conditions allowing the variant to be expressed and/or isolated. The cultivation takes place in a suitable nutrient medium comprising carbon and nitrogen sources and inorganic salts, using procedures known in the art. Suitable media are available from commercial suppliers or may be prepared according to published compositions (e.g., in catalogues of the American Type Culture Collection). If the variant is secreted into the nutrient medium, the variant can be recovered directly from the medium. If the variant is not secreted, it can be recovered from cell lysates.

The variant may be detected using methods known in the art that are specific for cutinases. These detection methods include, but are not limited to, use of specific antibodies, formation of an enzyme product, or disappearance of an enzyme substrate. For example, an enzyme assay may be used to determine the activity of the variant. Methods described in the present application may in some embodiments be suitable for this purpose.

The variant may be recovered using methods known in the art. For example, the variant may be recovered from the nutrient medium by conventional procedures including, but

not limited to, collection, centrifugation, filtration, extraction, spray-drying, evaporation, or precipitation. The variant may be purified by a variety of procedures known in the art including, but not limited to, chromatography (e.g., ion exchange, affinity, hydrophobic, chromatofocusing, and size exclusion), electrophoretic procedures (e.g., preparative isoelectric focusing), differential solubility (e.g., ammonium sulfate precipitation), SDS-PAGE, or extraction (see, e.g., Protein Purification, Janson and Ryden, editors, VCH Publishers, New York, 1989) to obtain substantially pure variants.

In an alternative aspect, the variant is not recovered, but rather a host cell of the present invention expressing the variant is used as a source of the variant.

Compositions and Forms

In some embodiments the invention relates to compositions comprising the variants. The composition may be a liquid, a gel, a powder, a granulate or any combination thereof.

In another embodiment the invention relates to variants which has been immobilized either on a carrier; by entrapment in natural or synthetic matrices, such as sol-gels, alginate, and carrageenan; by cross-linking methods such as in cross-linked enzyme crystals (CLEC) and cross-linked enzyme aggregates (CLEA); or by precipitation on salt crystals such as protein-coated micro-crystals (PCMC). Suitable carriers may be a hydrophilic carrier selected from the group containing: porous in-organic particles composed of alumina, silica and silicates such as porous glass, zeolites, diatomaceous earth, bentonite, vermiculite, hydrotalcite; and porous organic particles composed of carbohydrate polymers such as agarose or cellulose.

Methods and Uses

Cutinases may be used for synthetic polymer modification due to their ability to catalyze a number of important polymer biotransformations on poly(ethyleneterephthalate) (PET), Nylon 6,6, polyvinylacetate, polyacrylonitrile etc. Accordingly, increasing cutinase activity for selected polyester substrates may be utilized in various applications such as e.g.: (a) Surface modification of industrially important materials to tailor their physico-chemical properties which may be desirably for enhancing biocompatibility, chemical resistance, hydrophobicity, adhesion and wettability; (b) Improving the efficiency at which cutins in plant biomass are converted to chemical feedstocks; and (c) Developing enzymatic routes to recycle polyesters such as poly(omega-hydroxyfattyacids) and synthetic polyesters such as PET.

In one aspect the invention relates to a method of modifying a surface comprising: (a) adding the variant to a surface; and (b) detecting a change in at least one physico-chemical property, preferably wherein the at least one physico-chemical property is biocompatibility, chemical resistance, enhancing hydrophobicity, adhesion and/or wettability of the surface.

In one aspect the invention relates to a method of converting cutins in plant biomass to chemical feedstocks comprising: (a) adding the variant to a cutin containing plant biomass under conditions conducive for conversion.

In one aspect the invention relates to a method of recycling a polyester comprising: (a) adding the variant to the polyester to be recycled, preferably wherein the polyester is a poly(omega-hydroxyfattyacid) or a synthetic polyester.

In another aspect the invention relates to use of the variant for modification of a surface whereby at least one physico-chemical property is changed, preferably wherein the at least one physico-chemical property is any of biocompatibility,

chemical resistance, enhancing hydrophobicity, adhesion and/or wettability of the surface.

In another aspect the invention relates to use of the variant for improving the efficiency in converting cutins in plant biomass to chemical feedstocks.

In another aspect the invention relates to use of the variant for recycling a polyester, preferably wherein the polyester is a poly(omega-hydroxyfattyacid) or a synthetic polyester.

In some embodiments the invention relates to a method for obtaining a cutinase variant, comprising: (a) introducing into a parent cutinase substitution at one or more positions corresponding to positions 31, 36, 45, 52, 70, 120, 143, 167, and 174 of the mature polypeptide of SEQ ID NO: 2 selected from the group consisting of: P31A,R,N,D,C,Q,E,G,H,I,L,K,M,F,S,T,W,Y,V; 136A,R,N,D,C,Q,E,G,H,L,K,M,F,P,S,T,W,Y,V; G45A,R,N,D,C,Q,E,H,I,L,K,M,F,P,S,T,W,Y,V; N52E; F70A,R,N,D,C,Q,E,G,H,I,L,K,M,P,S,T,W,Y,V; G120S; G143A,R,N,D,C,Q,E,H,I,L,K,M,F,P,S,T,W,Y,V; L167A; and L174N,Q, wherein the variant has cutinase activity; and (b) recovering the variant.

Plants

The present invention also relates to plants, e.g., a transgenic plant, plant part, or plant cell, comprising a polynucleotide of the present invention so as to express and produce the variant in recoverable quantities. The variant may be recovered from the plant or plant part. Alternatively, the plant or plant part containing the variant may be used as such for improving the quality of a food or feed, e.g., improving nutritional value, palatability, and rheological properties, or to destroy an antinutritive factor.

The transgenic plant can be dicotyledonous (a dicot) or monocotyledonous (a monocot). Examples of monocot plants are grasses, such as meadow grass (blue grass, Poa), forage grass such as Festuca, Lolium, temperate grass, such as Agrostis, and cereals, e.g., wheat, oats, rye, barley, rice, sorghum, and maize (corn).

Examples of dicot plants are tobacco, legumes, such as lupins, potato, sugar beet, pea, bean and soybean, and cruciferous plants (family Brassicaceae), such as cauliflower, rape seed, and the closely related model organism *Arabidopsis thaliana*.

Examples of plant parts are stem, callus, leaves, root, fruits, seeds, and tubers as well as the individual tissues comprising these parts, e.g., epidermis, mesophyll, parenchyme, vascular tissues, meristems. Specific plant cell compartments, such as chloroplasts, apoplasts, mitochondria, vacuoles, peroxisomes and cytoplasm are also considered to be a plant part. Furthermore, any plant cell, whatever the tissue origin, is considered to be a plant part. Likewise, plant parts such as specific tissues and cells isolated to facilitate the utilization of the invention are also considered plant parts, e.g., embryos, endosperms, aleurone and seed coats.

Also included within the scope of the present invention are the progeny of such plants, plant parts, and plant cells.

The transgenic plant or plant cell expressing a variant may be constructed in accordance with methods known in the art. In short, the plant or plant cell is constructed by incorporating one or more expression constructs encoding a variant into the plant host genome or chloroplast genome and propagating the resulting modified plant or plant cell into a transgenic plant or plant cell.

The expression construct is conveniently a nucleic acid construct that comprises a polynucleotide encoding a variant operably linked with appropriate regulatory sequences required for expression of the polynucleotide in the plant or plant part of choice. Furthermore, the expression construct may comprise a selectable marker useful for identifying

plant cells into which the expression construct has been integrated and DNA sequences necessary for introduction of the construct into the plant in question (the latter depends on the DNA introduction method to be used).

The choice of regulatory sequences, such as promoter and terminator sequences and optionally signal or transit sequences, is determined, for example, on the basis of when, where, and how the variant is desired to be expressed. For instance, the expression of the gene encoding a variant may be constitutive or inducible, or may be developmental, stage or tissue specific, and the gene product may be targeted to a specific tissue or plant part such as seeds or leaves. Regulatory sequences are, for example, described by Tague et al., 1988, *Plant Physiology* 86: 506.

For constitutive expression, the 35S-CaMV, the maize ubiquitin 1, or the rice actin 1 promoter may be used (Franck et al., 1980, *Cell* 21: 285-294; Christensen et al., 1992, *Plant Mol. Biol.* 18: 675-689; Zhang et al., 1991, *Plant Cell* 3: 1155-1165). Organ-specific promoters may be, for example, a promoter from storage sink tissues such as seeds, potato tubers, and fruits (Edwards and Coruzzi, 1990, *Ann. Rev. Genet.* 24: 275-303), or from metabolic sink tissues such as meristems (Ito et al., 1994, *Plant Mol. Biol.* 24: 863-878), a seed specific promoter such as the glutelin, prolamin, globulin, or albumin promoter from rice (Wu et al., 1998, *Plant Cell Physiol.* 39: 885-889), a *Vicia faba* promoter from the legumin B4 and the unknown seed protein gene from *Vicia faba* (Conrad et al., 1998, *J. Plant Physiol.* 152: 708-711), a promoter from a seed oil body protein (Chen et al., 1998, *Plant Cell Physiol.* 39: 935-941), the storage protein napA promoter from *Brassica napus*, or any other seed specific promoter known in the art, e.g., as described in WO 91/14772. Furthermore, the promoter may be a leaf specific promoter such as the rbcS promoter from rice or tomato (Kyojuka et al., 1993, *Plant Physiol.* 102: 991-1000), the chlorella virus adenine methyltransferase gene promoter (Mitra and Higgins, 1994, *Plant Mol. Biol.* 26: 85-93), the aldP gene promoter from rice (Kagaya et al., 1995, *Mol. Gen. Genet.* 248: 668-674), or a wound inducible promoter such as the potato pin2 promoter (Xu et al., 1993, *Plant Mol. Biol.* 22: 573-588). Likewise, the promoter may be induced by abiotic treatments such as temperature, drought, or alterations in salinity or induced by exogenously applied substances that activate the promoter, e.g., ethanol, oestrogens, plant hormones such as ethylene, abscisic acid, and gibberellic acid, and heavy metals.

A promoter enhancer element may also be used to achieve higher expression of a variant in the plant. For instance, the promoter enhancer element may be an intron that is placed between the promoter and the polynucleotide encoding a variant. For instance, Xu et al., 1993, supra, disclose the use of the first intron of the rice actin 1 gene to enhance expression.

The selectable marker gene and any other parts of the expression construct may be chosen from those available in the art.

The nucleic acid construct is incorporated into the plant genome according to conventional techniques known in the art, including *Agrobacterium*-mediated transformation, virus-mediated transformation, microinjection, particle bombardment, biolistic transformation, and electroporation (Gasser et al., 1990, *Science* 244: 1293; Potrykus, 1990, *Bio/Technology* 8: 535; Shimamoto et al., 1989, *Nature* 338: 274).

Agrobacterium tumefaciens-mediated gene transfer is a method for generating transgenic dicots (for a review, see Hooykas and Schilperoort, 1992, *Plant Mol. Biol.* 19: 15-38)

and for transforming monocots, although other transformation methods may be used for these plants. A method for generating transgenic monocots is particle bombardment (microscopic gold or tungsten particles coated with the transforming DNA) of embryonic calli or developing embryos (Christou, 1992, *Plant J.* 2: 275-281; Shimamoto, 1994, *Curr. Opin. Biotechnol.* 5: 158-162; Vasil et al., 1992, *Bio/Technology* 10: 667-674). An alternative method for transformation of monocots is based on protoplast transformation as described by Omirulleh et al., 1993, *Plant Mol. Biol.* 21: 415-428. Additional transformation methods include those described in U.S. Pat. Nos. 6,395,966 and 7,151,204 (both of which are herein incorporated by reference in their entirety).

Following transformation, the transformants having incorporated the expression construct are selected and regenerated into whole plants according to methods well known in the art. Often the transformation procedure is designed for the selective elimination of selection genes either during regeneration or in the following generations by using, for example, co-transformation with two separate T-DNA constructs or site specific excision of the selection gene by a specific recombinase.

In addition to direct transformation of a particular plant genotype with a construct of the present invention, transgenic plants may be made by crossing a plant having the construct to a second plant lacking the construct. For example, a construct encoding a variant can be introduced into a particular plant variety by crossing, without the need for ever directly transforming a plant of that given variety. Therefore, the present invention encompasses not only a plant directly regenerated from cells which have been transformed in accordance with the present invention, but also the progeny of such plants. As used herein, progeny may refer to the offspring of any generation of a parent plant prepared in accordance with the present invention. Such progeny may include a DNA construct prepared in accordance with the present invention. Crossing results in the introduction of a transgene into a plant line by cross pollinating a starting line with a donor plant line. Non-limiting examples of such steps are described in U.S. Pat. No. 7151204.

Plants may be generated through a process of backcross conversion. For example, plants include plants referred to as a backcross converted genotype, line, inbred, or hybrid.

Genetic markers may be used to assist in the introgression of one or more transgenes of the invention from one genetic background into another. Marker assisted selection offers advantages relative to conventional breeding in that it can be used to avoid errors caused by phenotypic variations. Further, genetic markers may provide data regarding the relative degree of elite germplasm in the individual progeny of a particular cross. For example, when a plant with a desired trait which otherwise has a non-agronomically desirable genetic background is crossed to an elite parent, genetic markers may be used to select progeny which not only possess the trait of interest, but also have a relatively large proportion of the desired germplasm. In this way, the number of generations required to introgress one or more traits into a particular genetic background is minimized.

The present invention also relates to methods of producing a variant of the present invention comprising: (a) cultivating a transgenic plant or a plant cell comprising a polynucleotide encoding the variant under conditions conducive for production of the variant; and (b) recovering the variant.

The present invention is further described by the following examples that should not be construed as limiting the scope of the invention.

EXAMPLES

Example 1

Immobilization: The enzymes were immobilized on a Lewatit VP OC 1600 matrix at a ratio of 20 milligram enzyme per gram matrix. The matrix was prepared by washing with 98% ethanol followed by water. The enzyme solution was added to the washed matrix and incubated at 4° C. for 18 hours with shaking. After incubation the supernatant was resolved on an SDS-PAGE to ascertain the extent of immobilization. Enzyme aliquots collected before immobilization was used as control. The immobilized enzyme was dried in a dessicator under vacuum to remove all moisture. The dried immobilized enzyme was used for transacylation reactions. Control reactions were conducted under identical conditions without the addition of immobilized enzymes.

Transacylation reaction: The reaction was conducted in microfuge tubes (2 ml). Immobilized enzyme (20 mg) was added to acyl donor (100 mM) and acyl acceptor (1-propanol, 100 mM) in di-isopropyl ether. Dodecane (5 mM) was used as internal standard. The reaction mixture was incubated at 30° C. for 24 hr at 750 rpm. After completion of incubation the supernatant was transferred to a fresh micro centrifuge tube and diluted 10 times with di-isopropyl ether for GC analysis.

Gas chromatography: Gas chromatography was conducted on Agilent gas chromatograph (Model 7890) fitted with a DB-5 column and flame ionization detector. Sample (1 ul, split ratio of 1:10) was injected with the injector temperature maintained at 250° C. and the detector temperature at 280° C. During the run the initial column temperature set at 50° C. for 1 minute and increased to 150° C. with 5° C/min and from 150° C. to 250° C. with 10° C/min increment using hydrogen as the carrier gas.

All reactions were conducted with individual substrates and percentage conversion was calculated as the difference of the initial (control) and final (reaction) substrate concentration normalized with respect to internal standards.

Example 2

Reactions were conducted as described in Example 1 with the parent cutinase and the variants using four different substrates (acyl donors): methyl isobutyrate, methyl trimethylacetate, methyl crotonate, and methyl methacrylate.

TABLE 1

Activity of variants towards methyl isobutyrate, methyl trimethylacetate, methyl crotonate, and methyl methacrylate shown as a relative number in comparison with the activity of the parent cutinase (WT).

Cutinase	methyl isobutyrate	methyl trimethyl-acetate	methyl crotonate	methyl methacrylate
Wild Type (WT)	100	100	100	100
I36N	124	273	119	107
I36Q	121	273	125	159
I36S	117	209	19	96
G45N	140	236	0	22
G120S	131	291	175	59
L174N	117	264	113	74

TABLE 1-continued

Activity of variants towards methyl isobutyrate, methyl trimethylacetate, methyl crotonate, and methyl methacrylate shown as a relative number in comparison with the activity of the parent cutinase (WT).				
Cutinase	methyl isobutyrate	methyl trimethyl-acetate	methyl crotonate	methyl methacrylate
L174Q	136	145	125	26
A14P, I169A	107	9	19	0
A14P, N52E, I169A	86	273	113	170

The invention described and claimed herein is not to be limited in scope by the specific aspects herein disclosed, since these aspects are intended as illustrations of several aspects of the invention. Any equivalent aspects are intended to be within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims. In the case of conflict, the present disclosure including definitions will control.

SEQUENCE LISTING

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<160> NUMBER OF SEQ ID NOS: 2

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<212> TYPE: DNA
<213> ORGANISM: Humicola insolens
<220> FEATURE:
<221> NAME/KEY: CDS
<222> LOCATION: (109)..(690)

<400> SEQUENCE: 1

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gctggtgact cgaaccatac cccggccgct cctgaacttg ttgcccgg  cag ctg gga      117
                                     Gln Leu Gly
                                     1

gcc atc gag aac ggc ctt gag agc ggc agc gcc aac gcc tgc ccc gac      165
Ala Ile Glu Asn Gly Leu Glu Ser Gly Ser Ala Asn Ala Cys Pro Asp
      5                               10                               15

gcc atc ctg atc ttt gct cgc ggc tcg acc gag cca ggc aac atg ggc      213
Ala Ile Leu Ile Phe Ala Arg Gly Ser Thr Glu Pro Gly Asn Met Gly
      20                               25                               30                               35

atc acc gtc ggc cct gct ctc gcc aac ggc ctt gag tcc cac atc cgg      261
Ile Thr Val Gly Pro Ala Leu Ala Asn Gly Leu Glu Ser His Ile Arg
                                     40                               45                               50

aac atc tgg atc cag ggc gtc ggc ggc cct tac gac gcc gcg ctg gcc      309
Asn Ile Trp Ile Gln Gly Val Gly Gly Pro Tyr Asp Ala Ala Leu Ala
      55                               60                               65

acc aac ttc ctg ccg cgg ggc acc tcg cag gcc aac atc gac gag ggc      357
Thr Asn Phe Leu Pro Arg Gly Thr Ser Gln Ala Asn Ile Asp Glu Gly
      70                               75                               80

aag cgg ctg ttt gcg ctg gcc aac caa aag tgc ccc aac acg ccc gtc      405
Lys Arg Leu Phe Ala Leu Ala Asn Gln Lys Cys Pro Asn Thr Pro Val
      85                               90                               95

gtc gcc ggc ggg tac agc cag ggc gcg gcg ctc atc gct gcc gcc gtc      453
Val Ala Gly Gly Tyr Ser Gln Gly Ala Ala Leu Ile Ala Ala Ala Val
      100                               105                               110                               115

agc gag ctc agc ggc gcc gtc aag gag cag gtc aag ggc gtc gcc ctc      501
Ser Glu Leu Ser Gly Ala Val Lys Glu Gln Val Lys Gly Val Ala Leu
      120                               125                               130

ttc gga tac acc caa aac ctc cag aac cgt ggc ggc atc ccc aac tac      549
Phe Gly Tyr Thr Gln Asn Leu Gln Asn Arg Gly Gly Ile Pro Asn Tyr
      135                               140                               145

ccg cgc gag cgc acc aag gtg ttc tgc aac gtt ggc gac gcc gtc tgc      597
Pro Arg Glu Arg Thr Lys Val Phe Cys Asn Val Gly Asp Ala Val Cys
      150                               155                               160

acc ggc acg ctc atc atc acc ccg gcg cat ctg tcg tac acg atc gag      645
Thr Gly Thr Leu Ile Ile Thr Pro Ala His Leu Ser Tyr Thr Ile Glu
    
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-continued

165	170	175	
gcg cgc ggt gag gcc gcg agg ttc ctg cgg gat	gcg atc cgt gct	690	
Ala Arg Gly Glu Ala Ala Arg Phe Leu Arg Asp Arg Ile Arg Ala			
180	185	190	
taaatg			696

<210> SEQ ID NO 2
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 <213> ORGANISM: Humicola insolens

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1	5 10 15
Cys Pro Asp Ala Ile Leu Ile Phe Ala Arg Gly Ser Thr Glu Pro Gly	
	20 25 30
Asn Met Gly Ile Thr Val Gly Pro Ala Leu Ala Asn Gly Leu Glu Ser	
	35 40 45
His Ile Arg Asn Ile Trp Ile Gln Gly Val Gly Gly Pro Tyr Asp Ala	
	50 55 60
Ala Leu Ala Thr Asn Phe Leu Pro Arg Gly Thr Ser Gln Ala Asn Ile	
	65 70 75 80
Asp Glu Gly Lys Arg Leu Phe Ala Leu Ala Asn Gln Lys Cys Pro Asn	
	85 90 95
Thr Pro Val Val Ala Gly Gly Tyr Ser Gln Gly Ala Ala Leu Ile Ala	
	100 105 110
Ala Ala Val Ser Glu Leu Ser Gly Ala Val Lys Glu Gln Val Lys Gly	
	115 120 125
Val Ala Leu Phe Gly Tyr Thr Gln Asn Leu Gln Asn Arg Gly Gly Ile	
	130 135 140
Pro Asn Tyr Pro Arg Glu Arg Thr Lys Val Phe Cys Asn Val Gly Asp	
	145 150 155 160
Ala Val Cys Thr Gly Thr Leu Ile Ile Thr Pro Ala His Leu Ser Tyr	
	165 170 175
Thr Ile Glu Ala Arg Gly Glu Ala Ala Arg Phe Leu Arg Asp Arg Ile	
	180 185 190

Arg Ala

The invention claimed is:

1. An isolated variant of a parent cutinase, which comprises an amino acid sequence having at least 80% sequence identity to the mature polypeptide of SEQ ID NO: 2 wherein amino acid residue 36 is substituted with another amino acid, and further wherein the substitution consists of I36A, R, N, D, C, Q, E, G, H, L, M, F, P, S, T, W, Y or V; and which variant has cutinase activity and has improved catalytic property compared to the parent. 50
2. The variant of claim 1, wherein the cutinase activity is measured on short-chained alkyl ester substrates.
3. The variant of claim 1 having at least 90% sequence identity to the mature polypeptide of SEQ ID NO: 2. 60
4. The variant of claim 1, wherein the parent cutinase comprises or consists of the mature polypeptide of SEQ ID NO: 2.
5. The variant of claim 1, wherein the parent cutinase is a fragment of the mature polypeptide of SEQ ID NO: 2, wherein the fragment has cutinase activity. 65
6. The variant of claim 1, wherein the variant consists of 100 to 194, 150 to 194, 100 to 150, or 150 to 194 amino acids.
7. The variant of claim 1, which comprises or consists of at least one of the mutations/combination of mutations selected from: 136N, 136Q and 136S.
8. The variant of claim 1, which further comprises at least one substitution at a position corresponding to positions: G8D, A14P, E30Q, E47R, E47K, R51P, I169A, I169V, or E179Q of the mature polypeptide of SEQ ID NO: 2.
9. The variant of claim 1, which has an improved property relative to the parent, wherein the improved property is selected from the group consisting of catalytic efficiency, catalytic rate, specific activity, substrate binding, substrate cleavage, substrate specificity.
10. An isolated polynucleotide encoding the variant of any one of claim 1.
11. A host cell comprising the polynucleotide of claim 10.

12. A method of producing a cutinase variant, comprising:
(a) cultivating the host cell of claim **11** under conditions suitable for expression of the variant; and (b) recovering the variant.

13. A method of modifying a surface comprising: (a) 5
adding the variant of claim **1** to a surface; and (b) detecting a change in at least one physico-chemical property, preferably wherein the at least one physico-chemical property is biocompatibility, chemical resistance, enhancing hydrophobicity, adhesion and/or wettability of the surface. 10

14. A method of converting cutins in plant biomass to chemical feedstocks comprising: (a) adding the variant of claim **1** to a cutin containing plant biomass under conditions conducive for conversion.

15. A method of recycling a polyester comprising: (a) 15
adding the variant of claim **1** to the polyester to be recycled, preferably wherein the polyester is a poly(omega-hydroxy-fattyacid) or a synthetic polyester.

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